RAMAN AND SURFACE ENHANCED RAMAN OF BIOLOGICAL MATERIAL

Jason A. Guicheteau*[ab], Kristina Gonser[b], Steven D. Christesen[b],
[a]National Research Council Associate at the U.S. Army ECBC Research Lab, Aberdeen Proving Grounds Edgewood MD 21010.
[b]U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010.

ABSTRACT

In light of recent world events, an emphasis has been placed firmly on the development of rapid biological threat detection techniques for the military as well as homeland security. Vibrational spectroscopic methods such as Raman and surface enhanced Raman scattering (SERS) provide rapid detailed fingerprint information about the molecular composition of biomaterial in a non-destructive manner. The technology and methodology to acquire and interpret biological Raman spectra has seen tremendous advances over the past several decades. The problem of tissue fluorescence, which overwhelms the Raman signal of most biological samples upon excitation in the visible region, has been largely overcome by instrumentation operating in the near-IR region of the spectrum. In addition, treatment of biomaterials with silver nanoparticles not only enhances the normal Raman signal by several orders of magnitude but also further reduces the fluorescence backgrounds via interaction between the analyte and the metal SERS substrate. This work presents a study into the applicability of qualitative Raman spectroscopy with principal component analysis (PCA) and surface enhanced Raman for quantitative analysis of the biological threat materials associated with the Rapid Agent Aerosol Detection (RAAD) project.

1. INTRODUCTION

Raman spectroscopy is just one of the diverse set of detection techniques being evaluated for their ability to detect and identify biological materials. In order to compare and contrast different techniques, a Common Sample Set composed of threat simulants, interferents and growth media was provided to all RAAD participants. The samples were investigated using both normal and Surface Enhanced Raman Spectroscopy. This paper will focus on near-infrared Raman spectroscopy data from the Common Sample Set (CSS) bacteria. Results are also given from a Principal Component Analysis performed on both washed and unwashed bacteria. These measurements provide an initial assessment of the detection and discrimination capability of Raman spectroscopy as applied to biological materials. Despite the challenges facing this detection method, Raman spectroscopy is emerging as a rapid and information-rich method of investigating biological threats.

Numerous studies have been performed utilizing Raman and Surface enhanced Raman spectroscopy for the detection and analysis of biological materials (Manfait, 1997; Carron, 1999; Guzelian, 2002; Kneipp et al. 2002; Zeiri, 2004). It is nondestructive and does not require extrinsic contrast-enhancing agents. Further more Raman requires minimal sample preparation, and has acquisition times ranging from a few minutes down to seconds. For additional sensitivity, surface enhanced Raman scattering (SERS) greatly enhances the relatively weak normal Raman signal by several orders of magnitude. When SERS is applied to a problem, a SERS coating can capture an analyte, concentrate it and perhaps modify the analyte at the surface. Due to the localized enhancement only molecules within the particle plasmon field will exhibit an enhanced Raman signal, thus eliminating interference from contaminates, reagents or other components in a solution. SERS offers high sensitivity, large informational content, and the fact that water molecules do not interfere with measurements have made SERS appealing for studies of biological systems.

This paper will focus on Raman spectroscopy data from the RAAD CSS. Both normal Raman and SERS were used to investigate these samples using near-infra red (NIR) Raman instruments. Results are also given from a Principal Component Analysis (PCA) performed on several bacteria. Finally, some of the challenges facing this detection method are discussed.

2. METHODOLGY

2.1 Samples & Preparation

As stated, samples of biological materials and interferents were received through the RAAD program as part of the CSS. This paper focuses on results from the five primary threat simulants. A table of these samples is given below in Table 2.1. Samples were received in

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 DEC 2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Raman And Surface Enhanced Raman Of Biological Material	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **National Research Council Associate at the U.S. Army ECBC Research Lab, Aberdeen Proving Grounds Edgewood MD 21010; U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010.**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**5**	

individual vials in a lyophilized form. Each vial contained 2 mL of material, with a target concentration of $1e^7$-$1e^8$ organisms per mL. The vials were stored at 4°C. To reconstitute the samples, 2 mL of deionized water was injected into each vial. The vial was then vortexed for 10 seconds. Small glass balls included in the vials assisted in suspending the biologic material. This was followed by extraction of the supernatant into a 1.5 micro-centrifuge tube. Five microliters of each sample was pipetted onto an aluminum-coated microscope slide (ThermoElectron) and dried in a desiccator. This procedure was used to prepare samples for normal Raman data collection.

Table 2.1. Primary CSS samples

Sample	Strain	Growth Phase	Growth Media	Washing	Live/ Dead	Comments
Bacillus subtilis	globigii	Spore	TSB-Mod	Dil. Saline/Water	L	Anthrax simulant
Bacillus subtilis	globigii	Veg. Stat	TSB	None	L	Anthrax simulant
Brucella neotomae	23459	Brucella broth	Stationary	Dil. Saline/Water	D-heat	Brucella simulant
Erwinia herbicola	13329	TSB	Stationary	None	L	Yersina field
Yersinia rohdei	43380	TSB	Stationary	Dil. Saline/Water	L	Yersinia simulant
MS2 Phage	Dugway strain	E-coli	Stationary	None	L	Viral simulant

2.2 Silver Nanoparticles.

Silver nanoparticle suspensions were prepared following a modified procedure of Lee and Meisel (Lee, 1982). In a one-liter three neck round bottom flask, 90 mg of silver nitrate (99+ %, Aldrich) was dissolved in 500 mL of deionized water. A condenser (250 mm jacket length) was placed on the center neck of the flask. A 25 mL addition funnel, with 10 mL of an aqueous sodium citrate solution (1%) was attached to a second neck of the flask. The heating mantle was controlled by

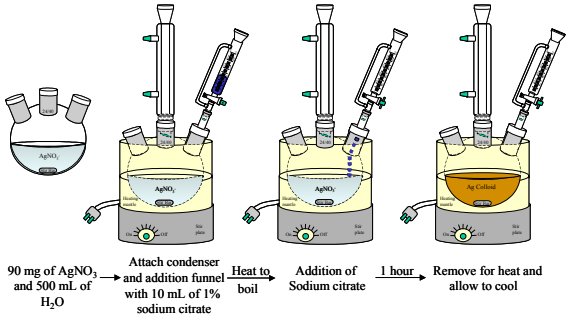

Figure 2.1 Nanoparticle Preparation

a 140-volt powerstat to slowly bring the silver nitrate solution to a boil. A stir bar was placed in the reaction flask and set to stir at a medium pace. In addition to above-mentioned set up, a heating top was placed around the flask to maintain temperature stability (Figure 2.1). To ensure batch-to-batch reproducibility of the nanoparticles, the speed of the stir plate between batches was kept identical. Once the solution boiled the sodium citrate was added rapidly. Heating and stirring was maintained for 1 hr after addition of the sodium citrate, at which time the reaction flask was removed from heat and allowed to cool to room temperature. The nanoparticle suspensions were yellowish-brown in appearance and the electronic absorption characterization of the colloids shows a λ_{max} of 400 nm.

Experiments carried out in solution were performed by adding 20 µL of the biologic of interest to 100 to 200 µL of colloidal solution. For samples prepared for slide analysis a similar preparation was carried out with the addition of dropping 5 µL of the colloid/biologic sample onto a slide and allowing to dry either in a incubator at 37°C or in a desiccator.

2.3 Instrumentation.

Two instruments were used to obtain spectra. Samples investigated in solution were analyzed with an EIC Echelle spectrograph fiber-optically coupled using an InPhotonics RamanProbe™ operating at 785 nm. Laser power was maintained at 160 mW and 20 second integration times were used.

For samples dried onto Aluminum slides, a Bruker Optics Senturion Raman Microscope operating at 785 nm was used. This system allowed the laser to be focused down onto samples. Collection occurred over a full spectral range (200-3200 cm^{-1}) with a resolution of 8-10 cm^{-1}. The microscope also had a video attachment, allowing visual imaging of the samples at powers of magnification up to 40x. Therefore, a very specific area of the sample could be targeted with the 5 µm laser spot. Typically, spectra were collected at 40x magnification and 20-second integration times. Laser power was adjusted between 0.177 mW and 5.3 mW to avoid damage to the samples. Data was collected in spc. format to be analyzed in Grams and Grams PLSplus-IQ software.

2.4 Principal Component Analysis

Data for the PCA was collected using the Raman microscope. Again, five microliters of each biologic were pipetted onto an Aluminum coated slide and dried in a desiccator overnight. Spectra were collected at 40x magnification, 30-second integration and a laser power maintained of 20 mW. *Bacillus subtilis* samples were photo-bleached as mentioned above. The sampling methods for each drop were performed starting from the top of the spot and moving left to right across the material, followed by repositioning the laser down from the last area and going back right to left and so forth. This was repeated until 50 spectra were collected. The collected spectra were analyzed in Grams PLSplus-IQ's principal component program (ThermoGalactic).

Spectra were automatically baselined and two regions, 800-1730 cm^{-1} and 2800-3030 cm^{-1} were used for constructing PCA plots.

3. RESULTS & DISCUSSION

3.1 Normal Raman

Representative Raman spectra for the threat simulants are show below in Figures 3.1 and 3.2. A moderate fluorescent background was observed for all

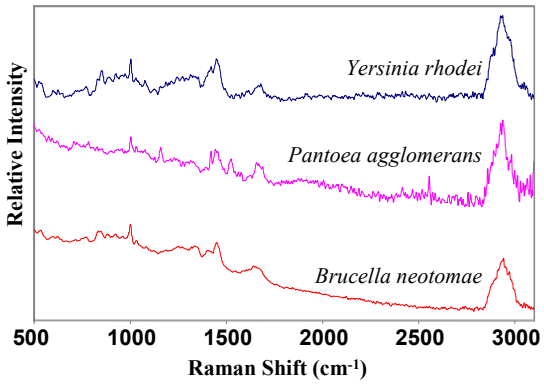

Figure 3.1 Sample spectra of three biologics.

samples. In the case of *Bacillus subtillis*, laser irradiation was used to photobleach the sample before data acquisition, resulting in a significantdecrease in fluorescent background.

All spectra display characteristic symmetric and antisymmetric CH_3 & CH_2 vibrations in the 2800 to

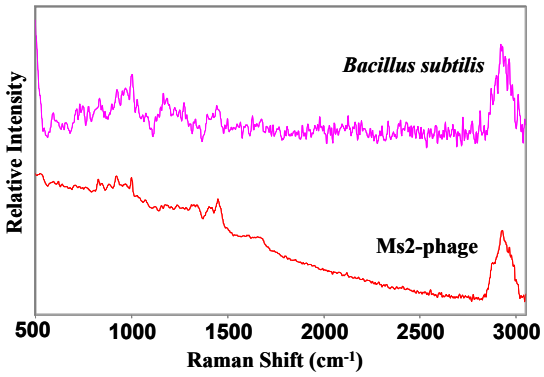

Figure 3.2 Sample spectra of biological material.

3000 cm^{-1} region. In addition, the spectra show other similarities. All spectra display a characteristic phenylalanine peak at approximately 1000 cm^{-1}. This peak was very weak in interefernts such as albumin. Additional characteristic biologic peaks are found in the region from 800-1730 cm^{-1}. *Yersinea rhodei, Erwinia herbolica,* and MS2 spectra display characteristic Amide I (~1620 to 1700 cm^{-1}) and Amide III (1220 and 1350 cm^{-1}) type vibrations in this area. These bands were very weak in *Bacillus subtillis*.

3.2 SERS

SERS experiments carried out on Al slides typically yielded superior spectral data than investigations carried out in solution. Figures 3.3, 3.4 were carried out on slides, while figure 3.5 is from solution. When compared to the normal Raman data collected, SERS showed improved signal-to-noise ratio, as well as improved spectral content resulting in additional peaks being identified that were previously masked by the background.

Figure 3.3 represents typical SERS spectra for *Pantoea agglomerans, Brucella neotomae,* and *Yersinia rhodei*. The highlighted regions show characteristic

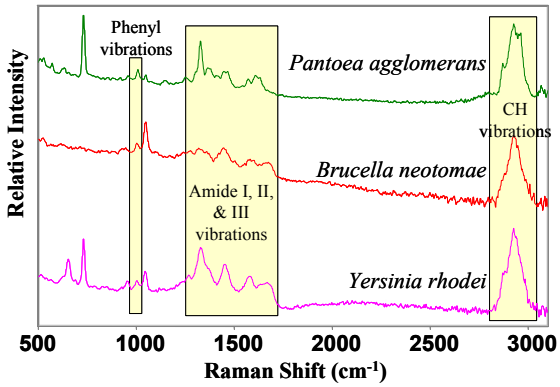

Figure 3.3 SERS spectra of biological material.

biological vibrations consisting of Amide I, II, & III vibrations between 1200 and 1680 cm^{-1}. The phenylalanine peak at 1001 cm^{-1} and the CH vibrations between 2280-3030 cm^{-1} are also observed.

Figure 3.4 shows a SERS spectrum of *Bacillus globigii* spores. The typical amide bands and CH

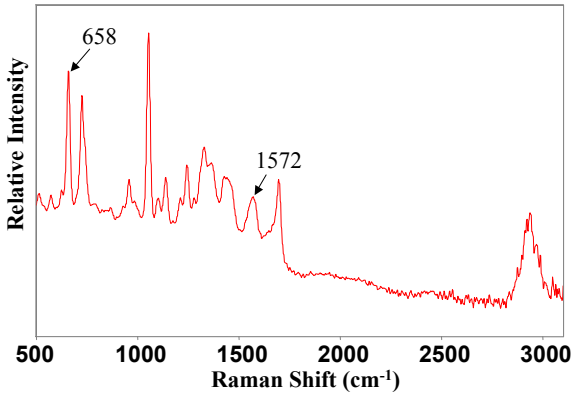

Figure 3.4 SERS spectrum of *Bacillus globigii* spores.

stretches are present with additional scattering from

calcium dipicolinate (CaDPA) which only spore forming bacteria contain. Several CaDPA peaks are observed at 658 cm^{-1} and 1578 cm^{-1}. A lot of interest has been generated recently focusing on the detection of CaDPA and its derivatives as a *Bacillus anthracis* signature (Esposito, 2003; Farquharson, 2004).

Figure 3.5 shows 4 spectra of *Bacillus subtillis*; however, the samples were in different media. Spectra A and B show samples grown in spent G media and spent sporulation broth, respectively. Spectrum C represents a vegetative sample from the "new" Dugway Proving

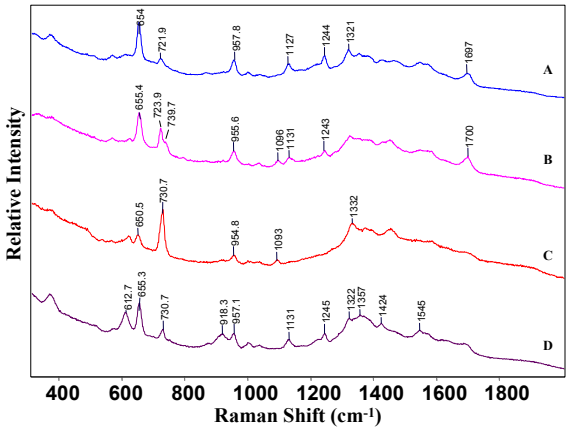

Figure 3.5 Spectra of *Bacillus subtilis* samples grown under different conditions

Ground (DPG) stock, while Spectrum D was taken from an "old" DPG grown in TSB media. While several peaks, such as 655 cm^{-1}, 730 cm^{-1}, 955 $cm^{-1}$and 1325 cm^{-1} are evident in all the spectra, others, such at 740 cm^{-1} in Spectrum B, are not. The increased sensitivity of SERS allows comparisons such as these to be made, allowing the discrimination of peaks characteristic to the organism and those that are only due to the growth media, or other changeable factor.

3.3 PCA

PCA is a data reduction and analysis technique that allows spectral similarities or differences to be easily seen. If the spectra of the biologics have definite, repeatable differences, the data points from each sample should cluster together, showing successful discrimination of a sample-specific Raman signature. qualitative analysis has been performed on *Bacillus subtilis, Pantoea agglomerans* and *Yersinia rohdei.* We have seen some discrimination between genera of bacteria, but as of yet are not able to distinguish species. Figure 3.6 shows a representative spectrum from the data set and the regions used for PCA analysis. The mid region, as it is labeled on the plot, is from 800 to 1730 cm^{-1} and includes among other vibrations, important Amide I and III modes that have been previously mentioned as characteristic of biomaterial. The region from 2800-3030 cm^{-1} contains varying types of CH stretching vibrations associated with organic chemicals as well as biomaterial. Figure 3.7 shows a principal component plot of PC1 versus PC2. The biomaterial was

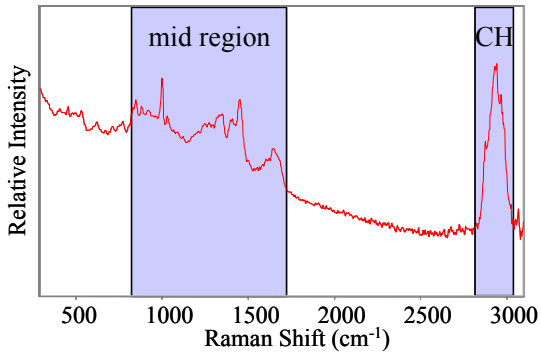

Figure 3.6 Representative spectrum used in PCA.

deposited onto an aluminum slide and excited by 785 nm light. Partial Least Square (PLS-IQ, Galactic) principal component program was used in the analysis. *Bacillus subtilis,* samples were photo bleached for 5 minutes to

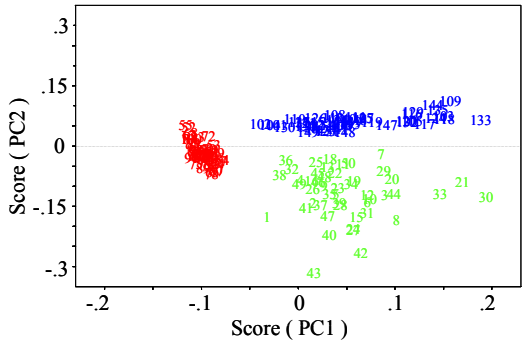

Figure 3.7 PCA plot showing separation of *Bacillus subtilis, Pantoea agglomerans,* and *Yersinia rhodei.*

reduce fluorescence and no other smoothing or baseline techniques were employed in performing the PCA. By increasing the sample set to include more biological threat material, a database can be created. This database can be used as a reference library to quickly identify potentially unknown biomaterial.

3.4 Challenges and further investigation

Despite encouraging results, there are several challenges to the practical application of Raman spectroscopy to the detection of biological threat agents. Biological materials have a low scattering cross-section, making it difficult to obtain the necessary sensitivity with a rapid response time. The ease of sample damage requires the use of low laser power, also contributing to a weaker signal. The use of SERS, however, can drastically improve the sensitivity and signal-to-noise ratio over normal Raman. High levels of fluorescence are also associated with biologics, which can effectively mask the weaker Raman peaks. A possible solution of

this problem is the use of ultraviolet wavelengths for the excitation laser. Below wavelengths of about 250 nm, the most fluorescence is greatly reduced. For this reason, future work will involve the collection of CSS data with a 248 nm ultraviolet laser as well as more extensive work with PCA and SERS. Finally, an ever-present challenge to developing a fieldable detector is the large and diverse number of interferents in the ambient environment. To this point, intereferents provided in the CSS have showed marked spectral differences from any of the biological threat simulants tested. Future experiments will continue to include biologics prepared under different conditions in an effort to truly isolate the signal from the organism from that of its growth media or other environmental factors.

4. CONCLUSIONS

In this paper, the ability of Raman spectroscopy to detect and discriminate biological material was discussed based on results from the RAAD CSS. Both normal and SERS spectra were presented and showed encouraging results. Based on the PCA, Raman spectroscopy has the potential not only to detect biologic particles, but also to correctly discriminate between several different biologics. At the same time, the technique has a rapid response time with little to no sample preparation needed for collection of data. Together with uniqueness of the spectra, these attributes continue to make Raman spectroscopy a viable candidate for inclusion in a rapid, sensitive biological detector

ACKNOWLEDGEMENTS

The authors would like to thank Cathy Scotto at NRL for her work in distributing the Common Sample Set, as well as the other members of the RAAD program for their helpful discussions. Also we would like to thank the Critical Reagent Program at the US Army ECBC for providing samples of *Bacillus subtilis*.

REFERENCES

Manfait, M., Charonov, S., Chourpa, I., Valisa, P. 1997: Confocal Raman Microscopic Imaging of Biological Materials, Proc. SPIE vol **2980**, 210-216.

Carron, K., Sulk R., Chan, C., Guicheteau, J., Gomez, C., Heyns, J., Corcoran, R. 1999: Surface Enhanced Raman Assays (SERIA): Measurment of Bilirubin and Salicylate. *J. Raman.* **30**, 853-859.

Guzelian, A., Sylvia, J., Janni, J., Clauson, L., Spencer, K., 2002: SERS of Whole Cell Bacteria and Trace Levels of Biological Molecules, Proc. SPIE vol **4577**, 182-192.

Kneipp, K., Kneipp, H., Itzkan, I., Dasari, R., Feld, M. 2002: Surface-Enhanced Raman Scattering and Biophysics. *J. Phys.: Condens. Matter*, **14,** R597-R624.

Zeiri, L., Bronk, B., Shabtai, Y., Eichler, J., Efrima, S. 2004: Surface-Enhanced Raman Spectroscopy as a Tool for Probing Specific Biochemical Components in Bacteria. *App. Spec.*, **58**(1), 33-40.

Lee, P. C., Meisel, D. 1982: *J. Phys. Chem,* **86**, 3391-3395.

Esposito, A., Talley, C., Huser, T., Hollars, C., Schaldach, C., Lane, S. 2003: Analysis of Sinle Bacterial Spores by Micro-Raman Spectroscopy, *App. Spec.,* 57(7), 868-870.

Farquharson, S., Gift, A., Maksymiuk, P., Inscore, F., 2004: Rapid Dipicolinic Acid Extraction from *Bacillus* Spores Detected by Surface-Enhanced Raman Spectroscopy. *App. Spec.,* **58**(3), 351-354.